



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : A61K 39/39</p>	<p>A1</p>	<p>(11) International Publication Number: WO 97/20576</p> <p>(43) International Publication Date: 12 June 1997 (12.06.97)</p>																				
<p>(21) International Application Number: PCT/GB96/03019</p> <p>(22) International Filing Date: 9 December 1996 (09.12.96)</p> <p>(30) Priority Data: 9525083.3 7 December 1995 (07.12.95) GB</p> <p>(71) Applicant (for all designated States except US): DANBIOSYST UK LIMITED (GB/GB); Albert Einstein Centre, Highfields Science Park, Nottingham NG7 2TN (GB).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): ILLUM, Lisbeth (DK/GB); 19 Cavendish Crescent North, The Park, Nottingham NG7 1BA (GB).</p> <p>(74) Agent: BASSETT, Richard; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>																				
<p>(54) Title: VACCINE COMPOSITIONS FOR INTRANASAL ADMINISTRATION COMPRISING CHITOSAN AND USE THEREOF</p> <div data-bbox="316 1134 1307 1774"> <table border="1"> <caption>Approximate Log10 Titre values from chart</caption> <thead> <tr> <th>Group</th> <th>Post 1st</th> <th>Post 2nd</th> <th>Post 3rd dose</th> </tr> </thead> <tbody> <tr> <td>PSA + chitosan I/N</td> <td>2.7</td> <td>4.6</td> <td>5.8</td> </tr> <tr> <td>PSA I/N</td> <td>1.8</td> <td>3.3</td> <td>3.3</td> </tr> <tr> <td>PSA SC</td> <td>3.3</td> <td>4.5</td> <td>5.8</td> </tr> <tr> <td>PSA daily (x3) I/N</td> <td>2.2</td> <td>2.3</td> <td>2.2</td> </tr> </tbody> </table> </div> <p>(57) Abstract</p> <p>There is provided vaccine compositions for intranasal administration, which compositions comprise one or more antigens and an effective adjuvant amount of a chitosan.</p>			Group	Post 1st	Post 2nd	Post 3rd dose	PSA + chitosan I/N	2.7	4.6	5.8	PSA I/N	1.8	3.3	3.3	PSA SC	3.3	4.5	5.8	PSA daily (x3) I/N	2.2	2.3	2.2
Group	Post 1st	Post 2nd	Post 3rd dose																			
PSA + chitosan I/N	2.7	4.6	5.8																			
PSA I/N	1.8	3.3	3.3																			
PSA SC	3.3	4.5	5.8																			
PSA daily (x3) I/N	2.2	2.3	2.2																			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

Vaccine compositions for intranasal administration comprising chitosan and use thereof

This invention relates to vaccine compositions for intranasal administration, which compositions comprise one or more antigens and
5 an effective adjuvant. The invention further relates to a method of immunising a mammal against diseases by administering such compositions to the mammal, methods of enhancing the immunogenicity of intranasally administered antigens, and uses of antigens in combination with an adjuvant for the manufacture of a vaccine composition for
10 intranasal administration to immunise a mammal against specific diseases.

Vaccines are preparations of antigenic materials, administered to recipients with a view to enhancing resistance to infection by inducing active immunity to specific microorganisms, for example bacteria or viruses.

15

Vaccines, which may be as single or mixed component vaccines, are presented in a variety of forms. For example, current influenza vaccines consist of either inactivated whole virus, disrupted virus (split vaccines) or purified preparations of antigenic proteins.

20

Vaccines are typically administered parenterally *via* injections. Traditional parenteral immunisation regimes are known to have a number of drawbacks. For example, many individuals possess a natural fear of injections and may experience psychological discomfort as a result.
25 Furthermore, many individuals find injections physically uncomfortable. Moreover, parenteral vaccination (e.g. intramuscular, sub-cutaneous etc.) is not an effective means of eliciting local antibody production if there has been no previous local exposure (e.g. by way of infection).

30 An effective local and/or topical administration regime is therefore

desirable.

In the case of some diseases, it would be advantageous to stimulate the mucosal immune system. In order to do this, the vaccine must be applied topically to a mucosal surface. Thus, in certain cases (e.g. in the case of infections of the upper respiratory tract), it would be beneficial to obtain more effective stimulation of the local mucosal immune system of the respiratory tract.

Accordingly, a number of attempts have been made to develop mucosal vaccines. One drawback, however, is that inactivated vaccines are often poorly immunogenic when given mucosally. In order to overcome this problem, different approaches to improving the immunogenicity of vaccines given orally or intranasally have included the use of adjuvants (see below), encapsulation of the vaccine in a variety of microspheres, and the use of live attenuated strains.

Certain adjuvants have been shown, when co-administered with vaccine antigens, to further boost the effectiveness of vaccine compositions by stimulating the immune response (see e.g. Hibberd *et al*, *Ann. Intern. Med.*, 110, 955 (1989)). Examples of adjuvants which have been shown to be effective include interferon alpha, *Klebsiella pneumoniae*, glycoprotein and interleukin-2.

Chitosans are derivatives of chitin or poly-N-acetyl-D-glucosamine in which the greater proportion of the N-acetyl groups have been removed through hydrolysis.

European Patent Application 460 020 discloses pharmaceutical formulations including chitosans as mucosal absorption enhancers. That

the chitosan could provide an adjuvant effect when administered in a vaccine composition is neither disclosed nor suggested.

We have now found surprisingly that, upon intranasal co-administration,
5 chitosan enhances the immune response of antigens and thus provides an adjuvant effect.

Accordingly, in a first aspect of the invention, there is provided a vaccine composition adapted for intranasal administration, which composition
10 comprises antigen and an effective adjuvant amount of a chitosan (hereinafter referred to as "the compositions according to the invention").

The term "effective adjuvant amount" will be well understood by those skilled in the art, and includes an amount of a chitosan which is capable
15 of stimulating the immune response to nasally administered antigens, i.e. an amount that increases the immune response of a nasally administered antigen composition, as measured in terms of the IgA levels in the nasal washings. Suitably effective increases in IgA levels include by more than 5%, preferably by more than 25%, and in particular by more than 50%,
20 as compared to the same antigen composition without any adjuvant.

Preferred concentrations of the chitosan in the compositions according to the invention are in the range 0.02 to 10%, more preferably 0.1 to 5% and particularly 0.25 to 2%.

25

We have found that, by administration of an antigen together with a particular chitosan derivative in an intranasal composition, it is possible to achieve an immune (e.g IgG and IgA) response. We have also found that, if a chitosan is incorporated into intranasal vaccine compositions
30 containing an antigen, good systemic and local immune responses are

produced. In particular, we have found that the intranasal administration of the compositions according to the invention enhances both a protective IgA mucosal immune response and an IgG systemic immune response.

5 Thus, the invention further provides a method of enhancing a protective IgA mucosal immune response and an IgG systemic immune response by administering intranasally to a mammal a vaccine composition comprising an antigen and an effective adjuvant amount of a chitosan.

10 The antigen may be provided as a sub-unit of a cell wall protein or polysaccharide, or as DNA which produces the antigen in the cells after introduction of the DNA (e.g. by transfection). Strictly speaking, the DNA is not itself an "antigen" but it encodes the antigen and is termed antigen herein.

15

The antigen may further be provided in a purified or an unpurified form. However, we prefer the antigen to be provided in a purified form.

The invention may be applied to antigens including proteins from
20 pathogens, recombinant proteins, peptides, polysaccharides, glycoproteins, lipopolysaccharides and DNA molecules (polynucleotides).

The following list of antigens is provided by means of illustration and is not meant to be exclusive: influenza virus antigens (such as
25 haemagglutinin and neuraminidase antigens), *Bordetella pertussis* antigens (such as pertussis toxin, filamentous haemagglutinin, pertactin), human papilloma virus (HPV) antigens, *Helicobacter pylori* antigens, rabies antigens, tick-borne encephalitis (TBE) antigens, meningococcal antigens (such as capsular polysaccharides of serogroup A, B, C, Y and W-135),
30 tetanus antigens (such as tetanus toxoid), diphtheria antigens (such as

diphtheria toxoid), pneumococcal antigens (such as *Streptococcus pneumoniae* type 3 capsular polysaccharide), tuberculosis antigens, human immunodeficiency virus (HIV) antigens (such as GP-120, GP-160), cholera antigens (such as cholera toxin B subunit), staphylococcal antigen
5 (such as staphylococcal enterotoxin B), shigella antigens (such as shigella polysaccharides), vesicular stomatitis virus antigen (such as vesicular stomatitis virus glycoprotein), cytomegalovirus (CMV) antigens, hepatitis antigens (such as hepatitis A (HAV), B (HBV), C (HCV), D (HDV) and G (HGV) virus antigens), respiratory syncytial virus (RSV) antigens,
10 herpes simplex antigens, or combinations thereof (e.g. combinations of diphtheria, pertussis and tetanus (DPT)). Suitable antigens also include those delivered for induction of tolerance, such as retinal antigens.

Preferred antigens include *Bordetella pertussis* antigens, meningococcal
15 antigens, tetanus antigens, diphtheria antigens, pneumococcal antigens, tuberculosis antigens and RSV antigens.

According to a further aspect of the invention, we prefer that the antigen
is not an influenza virus antigen.

20

Preferably, the chitosan is water-soluble, and may advantageously be produced from chitin by deacetylation to a degree of greater than 40%, preferably between 50% and 90%, and more preferably between 70% and 95%, deacetylation.

25

Particular deacetylated chitosans which may be mentioned include the "Sea Cure⁺" chitosan glutamate available from Protan Biopolymer A/S, Drammen, Norway.

30 The molecular weight of the chitosan may be between 10 kD and 500 kD,

preferably between 50 kD and 300 kD and more preferably between 100 kD and 300 kD.

The compositions according to the invention may be used in the
5 immunisation of a host against diseases, for example as described in the tests below.

According to a further aspect of the invention, there is provided a method of immunising a host against infection by disease, which method
10 comprises administering intranasally to the host a vaccine composition comprising antigen together with an effective adjuvant amount of a chitosan as hereinbefore defined.

Moreover, according to a further aspect of the invention, there is provided
15 a method of enhancing the immune response of an intranasally administered antigen, which method comprises co-administration of said antigen and a chitosan as hereinbefore defined.

The intranasal compositions according to the invention can be formulated
20 as liquids or dry powders, for administration as aerosols, drops or insufflations.

We prefer that the compositions according to the invention are formulated
as dry powders or in the form of microspheres.

25 Compositions for administration as nasal drops may contain one or more excipients of the type usually included in such compositions, for example preservatives, viscosity adjusting agents, tonicity adjusting agents, buffering agents and the like.

In order to ensure that the chitosan remains soluble in an aqueous medium, and to ensure also that the antigen is not adversely affected by too acidic a pH, a solution for intranasal administration preferably has a pH in the range 5.5 to 6.5, most preferably approximately pH 6.

5

The present invention also provides a means for dispensing the intranasal compositions of purified surface antigen and chitosan. A dispensing device may, for example, take the form of an aerosol delivery system, and may be arranged to dispense only a single dose, or a multiplicity of doses.

10

The vaccine will be administered to the patient in an amount effective to stimulate a protective immune response in the patient. For example, the vaccine may be administered to humans in one or more doses, each dose containing 1-250 micrograms and more preferably 2-50 micrograms of protein or polysaccharide antigen prepared from each viral or bacterial strain. For example, where haemagglutinin and neuraminidase preparations are prepared from three virus strains, e.g. 2 x Influenza A and 1 x Influenza B, a total dose of viral protein administered may be in the range 15-150 micrograms. Where *Bordetella pertussis* antigens are employed, a total dose of bacterial protein administered as FHA, pertussis toxin (toxoid) or pertactin, either individually or in combination may be in the range 5-150 micrograms.

15

20

The invention is illustrated, but in no way limited, by the following examples. The studies described clearly indicate that chitosan used as an adjuvant for mucosal vaccination, has the potential for enhancing both systemic and mucosal humoral responses to a number of antigens.

25

Brief Description of the Figures

Figure 1 illustrates the serum IgG anti-haemagglutinin response in mice immunised with purified surface antigen of influenza (PSA). Each bar represents the geometric mean titre of four mice. The error bars represent 1 standard error of the mean. The cut-off value is 50 which is the lower limit of detection.

Figure 2 illustrates the nasal IgA anti-haemagglutinin response in mice immunised with purified surface antigen (PSA). As with Figure 1, each bar represents the geometric mean titre of four mice, and the error bars represent 1 standard error or mean.

Figures 3a and 3b illustrate the determination of nasal and pulmonary anti-haemagglutinin secreting cells of mice immunised with purified surface antigen (PSA), using ELISPOT. Figure 3a uses a log scale whilst Figure 3b uses a linear scale.

Figures 4a, 4b and 4c illustrate the anti-*Bordetella pertussis* filamentous haemagglutinin (anti-FHA) serum IgG response, the anti-FHA secretory IgA response in lung lavage and the anti-FHA secretory IgA response, respectively, in nasal wash in mice immunised with FHA.

Figure 5a, 5b and 5c illustrate the anti-FHA and anti-Pertussis toxin (toxoid; anti-PT) serum IgG response, lung lavage secretory IgA response and the nasal wash secretory IgA response, respectively, in mice immunised with FHA and PT.

Example 1**Preparation of influenza B purified surface antigen/chitosan glutamate composition**

5 1A. A solution of 1% chitosan glutamate, a medium viscosity deacetylated chitin having approximately 11% residual N-acetyl groups, was prepared by dissolving the chitosan glutamate in 0.8% sodium chloride. The grade of chitosan glutamate used was "Sea Cure⁺ 210", available from Protan Biopolymer A/S, Drammen, Norway.

10

1B. Influenza purified surface antigen (PSA) containing both Influenza A and Influenza B protein, commercially available from Evans Medical Limited, Speke, Merseyside, United Kingdom, under the Trade Mark "Fluvirin", was made up in phosphate buffered saline to give a protein
15 concentration of approximately 1mg/ml. The PSA consists almost entirely of the spike protein haemagglutinin (HA), although it does contain some neuraminidase.

1C. A 1:1 mixture of the chitosan glutamate solution and the PSA
20 solution was prepared to give an intranasal vaccine composition containing 0.5% chitosan glutamate (11% acetylated), 0.8% NaCl, 0.05% PSA and phosphate buffer to give a solution pH of 6.

1D. Control solutions containing the same concentrations of PSA but not
25 chitosan glutamate, and the same concentrations of chitosan glutamate but no PSA, were also prepared. In addition, a composition comprising the same concentration of PSA adsorbed on to the known adjuvant Alhydrogel (aluminium hydroxide) was prepared. The PSA was adsorbed on to the Alhydrogel overnight at 40°C.

30

Example 2**Mice Immunisation Studies**

2A. The four compositions prepared as described in Example 1 were
5 administered to groups of twelve adult (6-8 weeks) female BALB/c mice
as follows:

- Group 1: 20 μ l (10 μ l per nostril) PSA/chitosan solution administered
intranasally. PSA dose = 10 μ g.
- 10 Group 2: 20 μ l PSA administered intranasally (total PSA dose = 10
 μ g).
- Group 3: 200 μ l PSA/Alhydrogel administered subcutaneously (PSA
dose = 10 μ g)
- Group 4: 20 μ l chitosan solution administered intranasally.
- 15 Group 5: 20 μ l PSA (10 μ l per nostril) administered daily for three
days. (Groups of four mice employed for this study).

2B. The immunisation procedure was carried out three times at monthly
intervals, with the exception of Group 5 where the mice were immunised
20 with three successive daily doses. The immunisation and sampling regime
is shown in Table 1.

Table 1

25

Immunisation	Day	Sample	Day
1	1	1	21
2	30	2	44
3	57	3	71+72

Immunisation and sampling regime

At each sampling point four mice from each group were terminally bled by cardiac puncture, their heads were removed and their nasal passages lavaged with 1 ml PBS + 1 % bovine serum albumin. Group 5 contained
5 four mice only so blood was obtained by tail puncture for the first two samples and nasal washes were only performed at the third sampling point.

Antibody assays

10 In all assays whole influenza vaccine (WIV) was used as antigen. Although WIV is only about 50% HA the assays were thought to be measuring primarily anti-HA antibodies. This assumption was confirmed by substituting PSA (100% HA) for WIV and repeating some assays. The results were similar with either antigen. HA-specific serum IgG and nasal
15 IgA antibodies were measured by Enzyme Linked Immunosorbant Assay (ELISA). After correcting for background, the individual optical density (OD) dilution curves were plotted and the titre values determined. The titre was determined as the dilution of serum that gave an OD reading of 0.2 or the dilution of nasal wash that gave an OD reading of 0.1.

20

As well as taking nasal washes at the third sample, lymphocytes were isolated from the mucous membranes of the nasal cavity and the lungs and the local immune response analysed by ELISPOT.

25 Results

1. Serum anti-HA serum response

Purified Surface Antigen (Figure 1 and Table 2):

30 As expected a good serum response was elicited by subcutaneous (SIC)

immunisation with PSA + Alhydrogel. All the animals tested had seroconverted after the primary immunisation and the geometric mean titre (GMT) was good. The response increased after each boost, the GMT after the third dose was very high (about 800,000). In contrast the serum response to PSA alone administered intranasally was poor: only two of four mice had seroconverted after the first dose, none of the mice tested had serum HA antibodies after the second dose (these are separate mice from those tested after the first immunisation) and although all animals tested had seroconverted after the third dose the GMT was lower than that of animals receiving one dose of PSA + Alhydrogel. Chitosan enhanced the serum response of intranasally administered PSA; after the third vaccination the antibody response in mice that received PSA + chitosan was 360-fold greater than that of mice receiving PSA alone I/N. The magnitude of the serum response in the PSA + chitosan mice was very similar to that of SIC immunised mice; in fact there was no statistical difference in the GMT's of the two groups at any sampling point (Student's t-Test $p > 0.01$).

Some mice were immunised three times on successive days with PSA alone administered intranasally to study whether this regime had advantages over the once monthly regime. Although all the mice in this group had detectable serum antibodies 21 days after the first dose and the GMT at this time point was greater than in mice that had received a single dose of PSA intranasally, the number of mice seropositive decreased during the course of the study although the GMT did not (in this group the same mice were sampled at each time point). At the final time point the GMT of the mice on the monthly regime was an order of magnitude greater than mice on the daily regime.

Table 2

Serum IgG anti-HA response in PSA immunised mice

Group	Post-Dose 1		Post-Dose 2		Post-Dose 3	
	Sero-Convsn. ^a	GMT	Sero-Convsn.	GMT	Sero-Convsn.	GMT
PSA + Chitosan	4/4	557	4/4	40504	4/4	653113
PSA I/N	2/4	67	0/4	< 50	4/4	1818
PSA S/C	4/4	2339	4/4	35196	4/4	816552
PSA 3 Daily Doses	4/4	182	3/4	229	2/4	180

^a No. positive/No. tested2. Nasal wash IgA anti-HA response

Purified Surface Antigen (Figure 2 and Table 3):

PSA + Alhydrogel given subcutaneously was very poor at inducing a nasal IgA response which is consistent with our previous findings and those of others. PSA alone given intranasally was also a poor mucosal immunogen although it was slightly better than subcutaneous immunisation in terms of the number of animals responding. Adding chitosan greatly boosted the IgA response, although the response was low after the first dose, HA-specific IgA could be detected in three out of four mice. The IgA response was boosted greatly in these mice by the second immunisation. The final immunisation had little effect; in fact the mean specific IgA levels had decreased slightly.

Table 3**Nasal IgA anti-HA response in PSA immunised mice**

Group	Post-Dose 1		Post-Dose 2		Post-Dose 3	
	Mucosal Convn.*	GMT	Mucosal Convn.	GMT	Mucosal Convn.	GMT
PSA + Chitosan	3/4	2.26	4/4	282.81	4/4	184.47
PSA I/N	0/4	< 1	1/4	1.20	3/4	2.31
PSA S/C	0/4	< 1	0/4	< 1	2/4	1.32
PSA 3 Daily Doses					0/4	< 1

* No. positive/No. tested

Responses to Chitosan Alone

The sera and nasal lavage fluid from the control mice immunised with chitosan alone were negative in all the assays.

Local anti-HA antibody secreting cell response (ASC) in nasal and pulmonary tissues

Lymphocytes were isolated from the nasal mucosa and lung parenchyma of groups of four mice at the third sampling point. Lymphocytes from individual mice were pooled and assayed for cells secreting IgA, IgG and IgM anti-flu antibodies using ELISPOT. The results are shown in Figures 3a and 3b.

B cells secreting HA-specific antibodies were detectable in the nasal and lung tissue of all groups. There were far greater numbers of such cells in

the PSA + chitosan group and this is most apparent when the results are plotted on a linear scale (Figure 3b). In all cases, except subcutaneously immunised mice, IgA antibody secreting cells (ASC) predominated in the nasal cavity whereas either IgG or IgM predominated in the lungs. The magnitude of the response is similar in the lungs and nose of PSA + chitosan mice.

Example 3

Preparation of *Bordetella pertussis* filamentous haemagglutinin/ chitosan glutamate composition

- 3A. A solution of 1% (10 mg/ml) chitosan glutamate (CSN) was prepared as described in Example 1 above.
- 3B *Bordetella pertussis* filamentous haemagglutinin (FHA), obtained from the National Institute for Biological Sciences, South Mimms, Potters Bar, London, United Kingdom, was made up in phosphate buffered saline (PBS) to give a protein concentration of approximately 1 mg/ml.
- 3C A 1:1 mixture of the CSN solution and the FHA solution was prepared to give an intranasal vaccine containing 0.5% CSN, 0.5 mg/ml FHA, 0.8% sodium chloride and phosphate buffer, pH 6.0.
- 3D Control solution for intranasal vaccination containing the same concentration of FHA but not CSN was also prepared. In addition, a mixture containing approximately 0.1 mg/ml of FHA adsorbed onto the known adjuvant Alhydrogel (aluminium hydroxide) was prepared. The FHA was adsorbed on to Alhydrogel by stirring the mixture for 30 minutes at room temperature.

Mice Immunisation Studies

3E The three compositions prepared as described above (sections 3C and 3D) were administered to groups of 13 adult male Balb/c mice as follows:

- Group 1: 20 μ l (10 μ l per nostril) FHA solution administered intranasally. (FHA dose = 10 μ g)
- Group 2: 20 μ l (10 μ l per nostril) FHA/CSN solution administered intranasally. (FHA dose = 10 μ g and chitosan = 100 μ g)
- Group 3: 50 μ l FHA/Alhydrogel mixture administered subcutaneously. (FHA dose = 5 μ g and Alhydrogel = 0.25 mg)

Immunisation and sampling regime

15

3D The immunisation procedure was carried out three times at monthly intervals. The immunisation and sampling regime is shown in Table 4.

3E Four mice from each group at sampling points 1 and 2 and five mice from each group at sampling point 3 were terminally bled by cardiac puncture. After collection of blood samples the animals were killed with an intravenous overdose of pentobarbitone sodium and the nasal passages and the lungs were respectively lavaged with 1 ml PBS containing 1% bovine serum albumin.

Table 4

Immunisation	Day	Sample	Day
1	1	1	21
2	28	2	42
3	56	3	70

Antibody analysis

3F Anti-FHA IgG antibodies in the serum samples and anti-FHA secretory IgA antibodies in the nasal wash and lung lavage samples were measured by Enzyme Linked Immunosorbant Assay (ELISA). To compare the antibody response elicited by the different compositions in the individual animals, certain values were assigned to the control positive serum and the control positive lung lavage samples, used for preparing the standard curves during the analysis of the test samples. The control positive serum and the control positive lung lavage were given values of 106 IgG Eq. units/ml and 104 IgA Eq. units/ml respectively.

Results

3G The serum IgG concentrations (Table 5 and Figure 4a) show that the nasally administered FHA/chitosan composition elicited a steady increase in response on days 22, 43 and 70. In comparison, the nasally administered FHA solution also elicited some response by Day 70, but this was only one fifth of the corresponding response produced by FHA/chitosan solution. As expected, the subcutaneously administered FHA/Alhydrogel composition elicited a high secondary response (Day 43).

This response was seven times as much as the corresponding response produced by the FHA/chitosan nasal composition. However, the response to subcutaneous administration decreased by Day 70 and this was only three times as much as the corresponding value for the FHA/chitosan nasal composition.

3H The secretory IgA concentrations in the lung lavages (Table 5, Figure 4b) clearly show that the FHA/chitosan nasal composition elicited the most response both on days 43 and 70 as compared to the responses produced by either the nasally administered FHA solution or the subcutaneously administered FHA/Alhydrogel composition. On Day 43 the response was 100 and 250 times as much as the responses elicited by the nasal FHA solution and the subcutaneous FHA/Alhydrogel composition respectively.

15

3I The secretory IgA concentrations in the nasal washes (Table 5 and Figure 4c) also show that the EHA/chitosan nasal composition elicited a steady increase in response on days 43 and 70. In comparison the subcutaneous administration produced no response at all, whereas the nasal FHA solution produced some response on Day 70, which was only a third of the response produced by the FHA/chitosan nasal composition.

20

Table 5

Serum IgG, lung lavage secretory IgA, and nasal wash secretory IgA antibody concentrations after the nasal administration of FHA (10 µg/mouse) either alone or in combination with chitosan in solution formulations and after the subcutaneous administration of FHA (5 µg/mouse) in combination with Alhydrogel in a solution formulation in mice.

Time after dosing (days)	Serum IgG (IgG Eq. units/ml ± SD)				Lung lavage IgA (IgA Eq. units/ml ± SD)				Nasal wash IgA (IgA Eq. units/ml ± SD)			
	FHA (nasal)	FHA/chitosan (nasal)	FHA/Alhydrogel (s.c.)		FHA (nasal)	FHA/chitosan (nasal)	FHA/Alhydrogel (s.c.)		FHA (nasal)	FHA/chitosan (nasal)	FHA/Alhydrogel (s.c.)	
22	242	3167	30092		78	28	44		0	42	0	
	±74.0	±3621.0	±9447.2		±56.8	±56.5	±4.2			±83.2		
Seroconversion	4	4	4		3	1	3		0	1	0	
43	4313	257720	1915684		423	46380	185		124	1657	0	
	±5912.2	±59170.6	±1706830.7		±507.9	±57554.3	±219.1		±144.4	±612.7		
Seroconversion	4	4	4		4	4	4		2	4	0	
70	92925	430139	1230048		4619	38006	87		918	2868	0	
	±162364.3	±121006.5	±687737.1		±8982.8	±48405.3	±20.5		±1527.1	±1503.9		
Seroconversion	5	5	5		3	5	5		2	5	0	

NOTE: For control IgG assume: 1 000 000 IgG Eq. units/ml
For control IgA assume: 10 000 IgA Eq. units/ml

Number of samples analysed per formulation for days 22 and 43 were 4

Number of samples analysed per formulation for day 70 were 5

Seroconversion: number of animals which produced a positive response (2.5 x background value) in each group

Example 4**Preparation of *Bordetella pertussis* filamentous haemagglutinin/pertussis toxin (toxoid)/chitosan glutamate composition**

5 4A. A solution of 2% (20 mg/ml) chitosan glutamate (CSN) was prepared by dissolving 200 mg chitosan in 10 ml water.

4B *Bordetella pertussis* filamentous haemagglutinin (FHA), obtained from CAMR, Salisbury, Wiltshire, United Kingdom, was concentrated
10 using an Aquacide II column and made up in phosphate buffered saline (PBS) to give a protein concentration of 267 $\mu\text{g/ml}$. Pertussis toxin (PT), non-toxic mutant, obtained from IRIS, Siena, Italy was concentrated using an Aquacide II column and made up in 0.5 M sodium chloride to give a final concentration of 267 $\mu\text{g/ml}$. Equal volumes of these two antigen
15 solutions were mixed to give a solution containing each antigen at a final protein concentration of 133 $\mu\text{g/ml}$.

4C A 1:3 mixture of 2% CSN solution and FHA/PT solution was prepared to give an intranasal vaccine containing 0.5% CSN,
20 approximately 100 $\mu\text{g/ml}$ FHA, 100 $\mu\text{g/ml}$ PT, 0.8% sodium chloride and phosphate buffer.

4D A control solution for intranasal vaccination containing the same concentration of FHA and PT but not CSN was prepared. A negative
25 control solution for intranasal vaccination containing 0.5% CSN but neither FHA nor PT was also prepared by diluting one part 2% CSN solution with three parts PBS containing 0.0014 M potassium chloride and 0.3185 M sodium chloride. In addition, a mixture containing approximately 10 $\mu\text{g/ml}$ FHA and 10 $\mu\text{g/ml}$ PT adsorbed onto the known
30 adjuvant Alhydrogel (200 ~g/ml) was prepared. The antigens were

adsorbed on to Alhydrogel by stirring the mixture for 30 min at room temperature.

Mice Immunisation Studies

5

4E The four compositions prepared as described above (sections 3C and 3D) were administered to groups of 10 adult female Balb/c mice as follows:

- 10 Group 1: 20 μ l (10 μ l per nostril) FHA/PT/CSN solution administered intranasally. (FHA dose = 2 μ g, PT dose = 2 μ g, chitosan dose = 100 μ g)
- Group 2: 20 μ l (10 μ l per nostril) PT/FHA solution administered intranasally. (FHA dose = 10 μ g, PT dose = 2 μ g)
- 15 Group 3: 20 μ l (10 μ l per nostril) CSN solution administered intranasally (CSN dose = 100 μ g)
- Group 4: 200 μ l FHA/PT/Alhydrogel mixture administered intraperitoneally. (FHA dose = 2 μ g, PT dose = 2 μ g, Alhydrogel = 40 μ g)

20

Immunisation and sampling regime

4D The immunisation procedure was carried out twice at a monthly interval. The immunisation and sampling regime is shown in Table 6.

25

4E Five mice from each group at sampling points 1 and 2 were terminally bled by cardiac puncture. After collection of blood samples the animals were killed with an intravenous overdose of pentobarbitone sodium and the nasal passages and the lungs were respectively lavaged

30 with 1 ml PBS containing 1 % bovine serum albumin.

Table 6

Immunisation	Day	Sample	Day
1	1	1	28
2	28	2	42

Antibody analysis

4F Anti-FHA IgG, anti-PT IgG antibodies in the serum samples and anti-FHA secretory IgA, anti-PT secretory IgA antibodies in the nasal wash and lung lavage samples were measured by Enzyme Linked Immunosorbant Assay (ELISA). The test samples were appropriately diluted with the sample buffer to four different concentrations and analysed in duplicate for each antibody. Titration curves were produced, the y-axis value was fixed at 2.5 times the background value and the interpolation value for each sample was calculated using the Kineticalc programme KC3. The geometric mean titration (GMT) value for each sample was then obtained by calculating the inverse of the interpolation value.

Results

4G The GMT values for both anti-FHA and anti-PT serum IgG antibodies (Tables 7, 8 and Figure 5a) show that the intranasally administered FHA/PT/chitosan composition (Group 1) elicited a primary systemic response and a considerably enhanced secondary response. In comparison, the nasally administered FHA/PT solution (Group 2) also elicited some primary systemic response and an enhanced secondary

response. However the secondary responses to both FHA and PT produced by Group 1 were approximately 5 fold higher than the corresponding responses produced by Group 2.

5 4H As expected, the intraperitoneally administered FHA/PT solution (Group 4) elicited high primary and secondary responses to FHA and PT (Tables 7, 8 and Figure 5A). However, the secondary responses to FHA and PT were approximately 30 and 10 fold higher than the primary responses respectively. The negative control group serum samples (Group
10 3) were found to be negative both for anti-FHA and anti-PT IgG antibodies (Tables 7, 8).

4I The GMT values for secretory IgA antibodies in the lung lavages (Tables 7, 8 and Figure 5b) clearly show that both the nasal compositions
15 FHA/PT/chitosan (Group 1) and FHA/PT (Group 2) elicited a secondary response to FHA and PT but neither antigen produced a primary response. The secondary responses to PT produced by Group 1 and Group 2 were similar, whereas this response to FHA produced by Group 1 was approximately 15 fold higher than the response to FHA produced by
20 Group 2.

4J The GMT values for secretory IgA antibodies in the nasal washes (Tables 7, 8 and Figure 5c) also show that both the nasal compositions in Group 1 and Group 2 elicited a secondary response to both the antigens,
25 but only PT produced a small primary response. The secondary response to FHA produced by Group 1 was almost 15 fold higher than that produced by Group 2, whereas the corresponding response to PT produced by Group 1 was only twice as much as that produced by Group 2.

30 4K The lung lavage and nasal wash samples from the chitosan control

group (Group 3) were found to be negative both for anti-FHA and anti-PT IgA antibodies (Tables 7, 8). The intraperitoneally administered FHA/PT/Alhydrogel (Group 4) was also found to produce no mucosal response (IgA antibodies) to either antigen in the lung lavage or the nasal washes.

Table 7
Summary of Geometric mean titration (GMT) values of anti-FHA serum IgG and anti-FHA secretory IgA (lung lavage and nasal wash) antibodies after the intraperitoneal or nasal administration of FHA (2 µg/mouse) and PT (2 µg/mouse) in various solution formulations in mice

Formulation	Day 28				Day 42				
	Serum anti-FHA IgG		Lung lavage anti-FHA IgA		Serum anti-FHA IgG		Lung lavage anti-FHA IgA		
	Seroconversion	Mean GMT value	SD	Seroconversion	Mean GMT value	SD	Seroconversion	Mean GMT value	SD
FHA/PT/chitosan/nasal (Group 1)	4 out of 5	499.5	388.1	0 out of 5	0.0	0.0	0 out of 5	0.0	0.0
FHA/PT/nasal (Group 2)	3 out of 5	77.8	83.6	0 out of 5	0.0	0.0	0 out of 5	0.0	0.0
chitosan/nasal (Group 3)	0 out of 5	0.0	0.0	0 out of 5	0.0	0.0	0 out of 5	0.0	0.0
FHA/PT/i.p. (Group 4)	4 out of 4	25140.2	13690.5	0 out of 4	0.0	0.0	0 out of 4	0.0	0.0
Formulation	Serum anti-FHA IgG		Lung lavage anti-FHA IgA		Serum anti-FHA IgG		Lung lavage anti-FHA IgA		
	Seroconversion	Mean GMT value	SD	Seroconversion	Mean GMT value	SD	Seroconversion	Mean GMT value	SD
	Seroconversion	Mean GMT value	SD	Seroconversion	Mean GMT value	SD	Seroconversion	Mean GMT value	SD
FHA/PT/chitosan/nasal (Group 1)	5 out of 5	118257.4	59051.0	4 out of 5	926.7	882.9	5 out of 5	19.6	14.1
FHA/PT/nasal (Group 2)	4 out of 5	25579.6	41735.6	2 out of 5	62.8	88.4	1 out of 5	1.3	2.9
chitosan/nasal (Group 3)	0 out of 5	0.0	0.0	0 out of 5	0.0	0.0	0 out of 5	0.0	0.0
FHA/PT/i.p. (Group 4)	5 out of 5	869421.2	419236.5	0 out of 5	0.0	0.0	0 out of 5	0.0	0.0

Seroconversion: number of animals which produced a positive response (2.5 x background optical density) in each group

Table 3
Summary of Geometric mean titration (GMT) values of anti-PT serum IgG and anti-PT secretory IgA (lung lavage and nasal wash) antibodies after the intraperitoneal or nasal administration of FHA (2 µg/mouse) and PT (2 µg/mouse) in various solution formulations in mice

Formulation	Day 28				Day 42			
	Seroconversion	Serum anti-PT IgG Mean GMT value	SD	Lung lavage anti-PT IgA Seroconversion Mean GMT value	SD	Seroconversion	Serum anti-PT IgG Mean GMT value	SD
FHA/PT/chitosan/nasal (Group 1)	5 out of 5	11471.1	7875.0	0 out of 4	0.0	5 out of 5	667556.6	748959.0
FHA/PT/nasal (Group 2)	5 out of 5	8794.4	10759.4	1 out of 5	0.2	5 out of 5	147081.3	134929.0
chitosan/nasal (Group 3)	0 out of 5	0.0	0.0	0 out of 5	0.0	0 out of 5	0.0	0.0
FHA/PT/i.p. (Group 4)	4 out of 4	19269.3	14648.3	0 out of 4	0.0	5 out of 5	220118.2	83448.9
Formulation	Day 28				Day 42			
	Seroconversion	Nasal wash anti-PT IgA Mean GMT value	SD	Lung lavage anti-PT IgA Seroconversion Mean GMT value	SD	Seroconversion	Nasal wash anti-PT IgA Mean GMT value	SD
FHA/PT/chitosan/nasal (Group 1)	4 out of 5	3.9	4.5	0 out of 4	0.0	5 out of 5	29.2	9.7
FHA/PT/nasal (Group 2)	3 out of 5	2.2	2.4	1 out of 5	0.5	5 out of 5	14.9	6.6
chitosan/nasal (Group 3)	0 out of 5	0.0	0.0	0 out of 5	0.0	0 out of 5	0.0	0.0
FHA/PT/i.p. (Group 4)	0 out of 4	0.0	0.0	0 out of 4	0.0	0 out of 5	0.0	0.0

Seroconversion: number of animals which produced a positive response (2.5 x background optical density) in each group

Claims

1. A vaccine composition adapted for intranasal administration, which composition comprises antigen and an effective adjuvant amount of a
5 chitosan.
2. A vaccine composition according to Claim 1 where the antigen is a protein from a pathogen, a recombinant protein, a glycoprotein, a peptide, a polysaccharide, a lipopolysaccharide or a polynucleotide.
10
3. A vaccine composition according to Claim 1 where the antigen is a whole cell or a virus.
4. A vaccine composition according to Claim 1 where the antigen is
15 provided as DNA which encodes the antigen.
5. A vaccine composition according to Claim 1 or Claim 2, wherein the antigen is a *Bordetella pertussis* antigen, a meningococcal antigen, a tetanus antigen, a diphtheria antigen, a pneumococcal antigen, a
20 tuberculosis antigen or a RSV antigen.
6. A vaccine composition according to Claim 1 or Claim 2, wherein the antigen is one which is delivered for induction of tolerance.
- 25 7. A vaccine composition according to Claim 1 or Claim 2, provided that the antigen is not an influenza virus antigen.
8. A vaccine composition according to any one of the preceding claims wherein the antigen is present in a purified form.
30

9. A vaccine composition according to any one of the preceding claims wherein the concentration of the chitosan is in the range 0.02 to 10%.
10. A vaccine composition as claimed in Claim 9 wherein the
5 concentration of the chitosan is in the range 0.1 to 5%.
11. A vaccine composition as claimed in Claim 10 wherein the concentration of the chitosan is in the range 0.25 to 2%.
- 10 12. A vaccine composition according to any one of the preceding claims wherein the chitosan is water soluble.
13. A vaccine composition according to any one of the preceding claims wherein the chitosan is produced from chitin by deacetylation to a degree
15 of greater than 40% deacetylation.
14. A vaccine composition as claimed in Claim 13 wherein the degree of deacetylation is between 50% and 90%.
- 20 15. A vaccine composition as claimed in Claim 14 wherein the degree of deacetylation is between 70% and 95%.
16. A vaccine composition according to any one of the preceding claims wherein the molecular weight of the chitosan is between 10 kD and 500
25 kD.
17. A vaccine composition as claimed in Claim 16 wherein the molecular weight of the chitosan is between 50 kD and 300 kD.
- 30 18. A vaccine composition as claimed in Claim 17 wherein the

molecular weight of the chitosan is between 100 kD and 300 kD.

19. A vaccine composition according to any one of the preceding claims wherein the composition has a pH in the range 5.5 to 6.5.

5

20. A vaccine composition as claimed in Claim 19 wherein the pH is approximately pH6.

21. A vaccine composition according to any one of the preceding claims
10 which is formulated as a dry powder or in the form of microspheres.

22. A pharmaceutical product comprising a dispensing device adapted to deliver a composition intranasally, in combination with a vaccine composition as defined in any one of the preceding claims.

15

23. A pharmaceutical product according to Claim 22 wherein the dispensing device is an aerosol delivery system.

24. A method of immunising a host against infection with a disease,
20 which method comprises administering intranasally to the host, a vaccine composition comprising the appropriate antigen together with an effective adjuvant amount of a chitosan as defined in any one of Claims 1 to 21.

25. A method of enhancing a protective IgA mucosal immune response
25 and an IgG systemic response by administering intranasally to a mammal a vaccine composition comprising an antigen and an effective adjuvant amount of a chitosan as defined in any one of Claims 1 to 21.

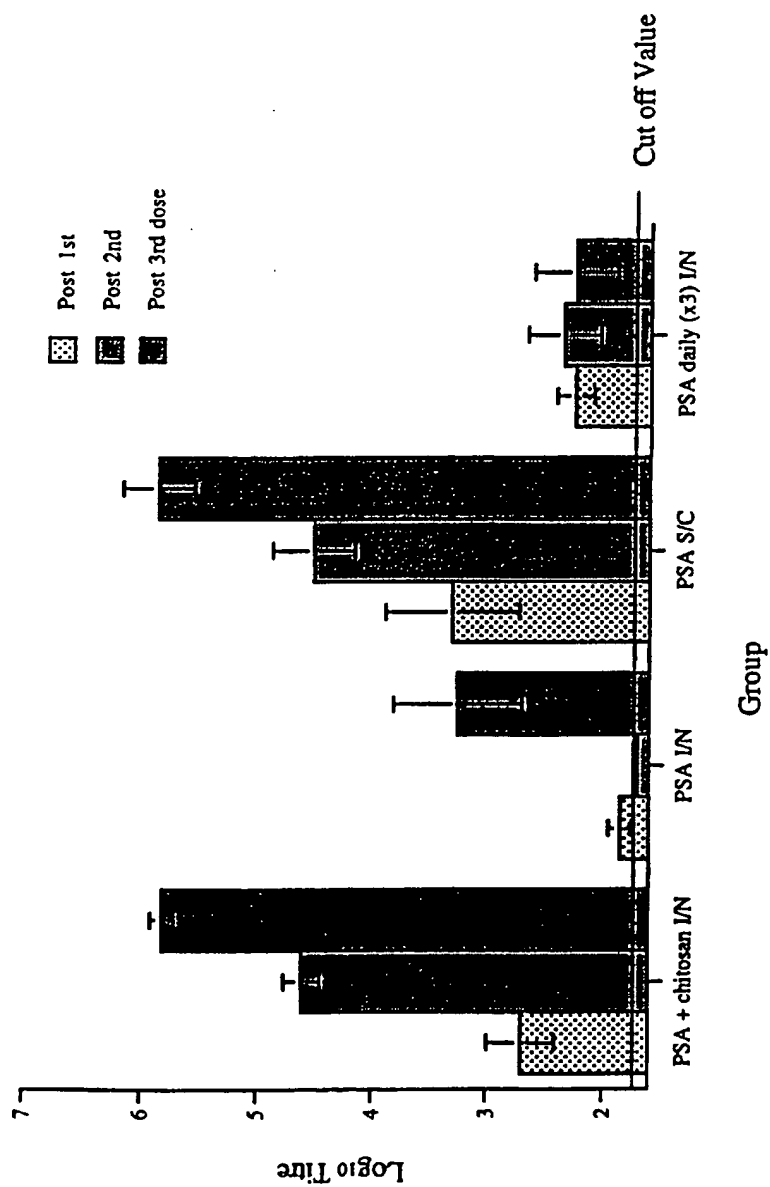
26. A method of enhancing the immune response of an intranasally
30 administered antigen, which method comprises co-administration of

antigen and a chitosan as defined in any one of Claims 1 to 21.

27. The use of a chitosan as defined in any one of Claims 1 to 21 for the manufacture of an intranasal adjuvant composition for enhancing the immunogenicity of intranasally administered antigens.
- 5

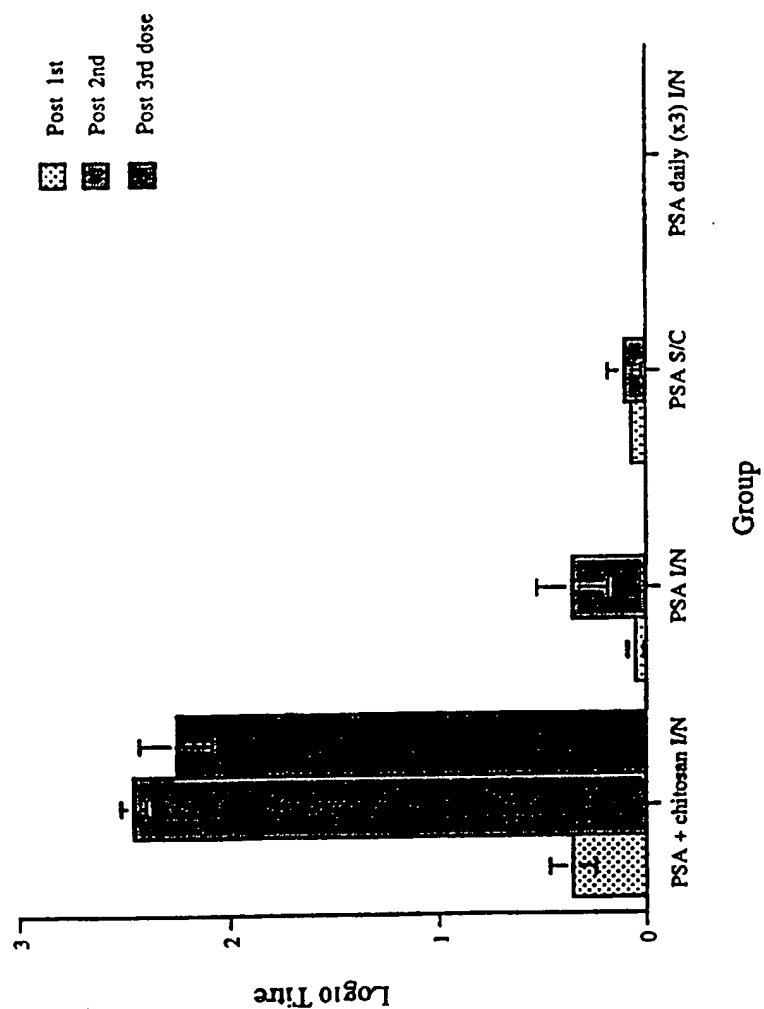
1/6

FIG. 1

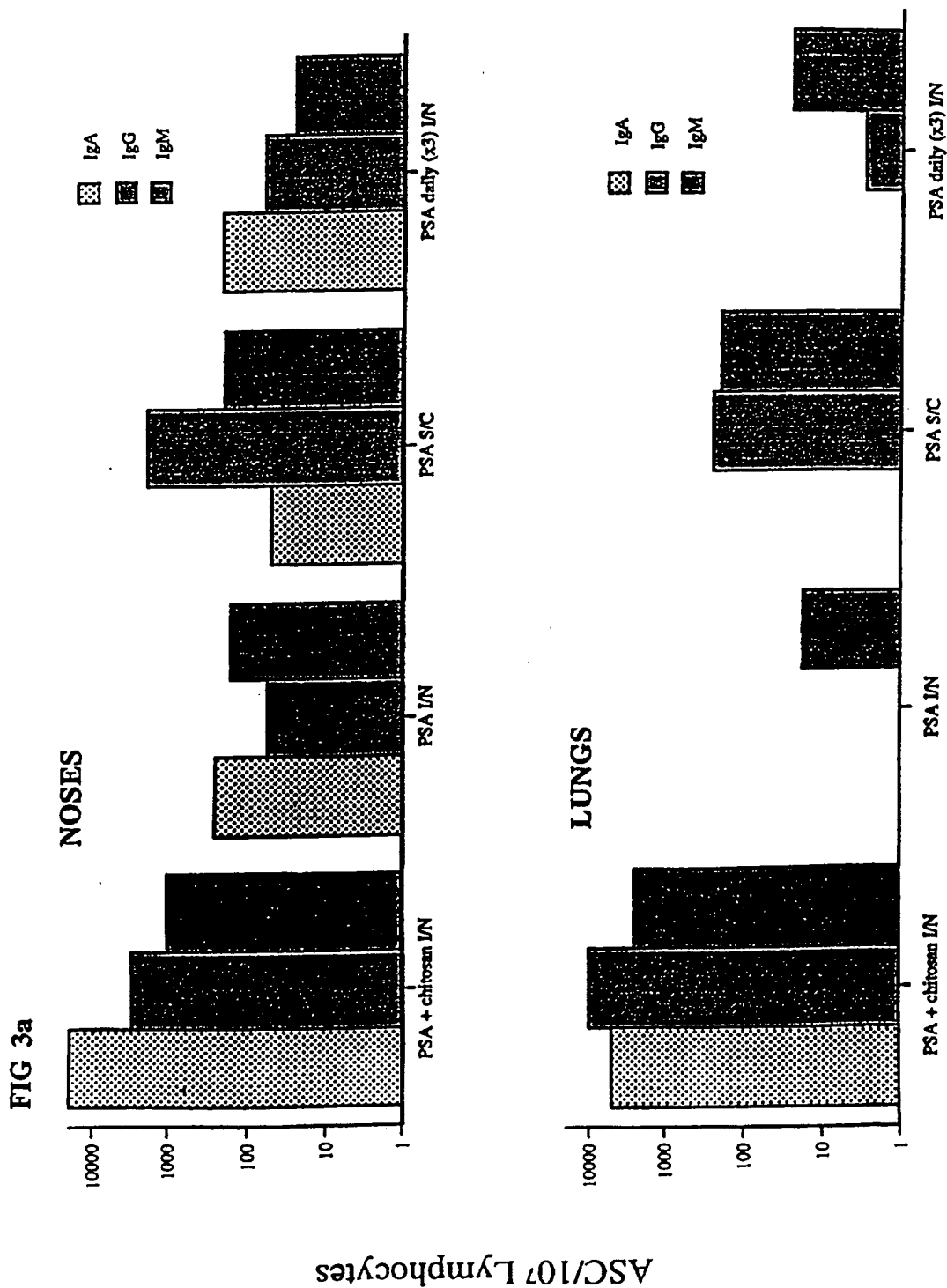


2/6

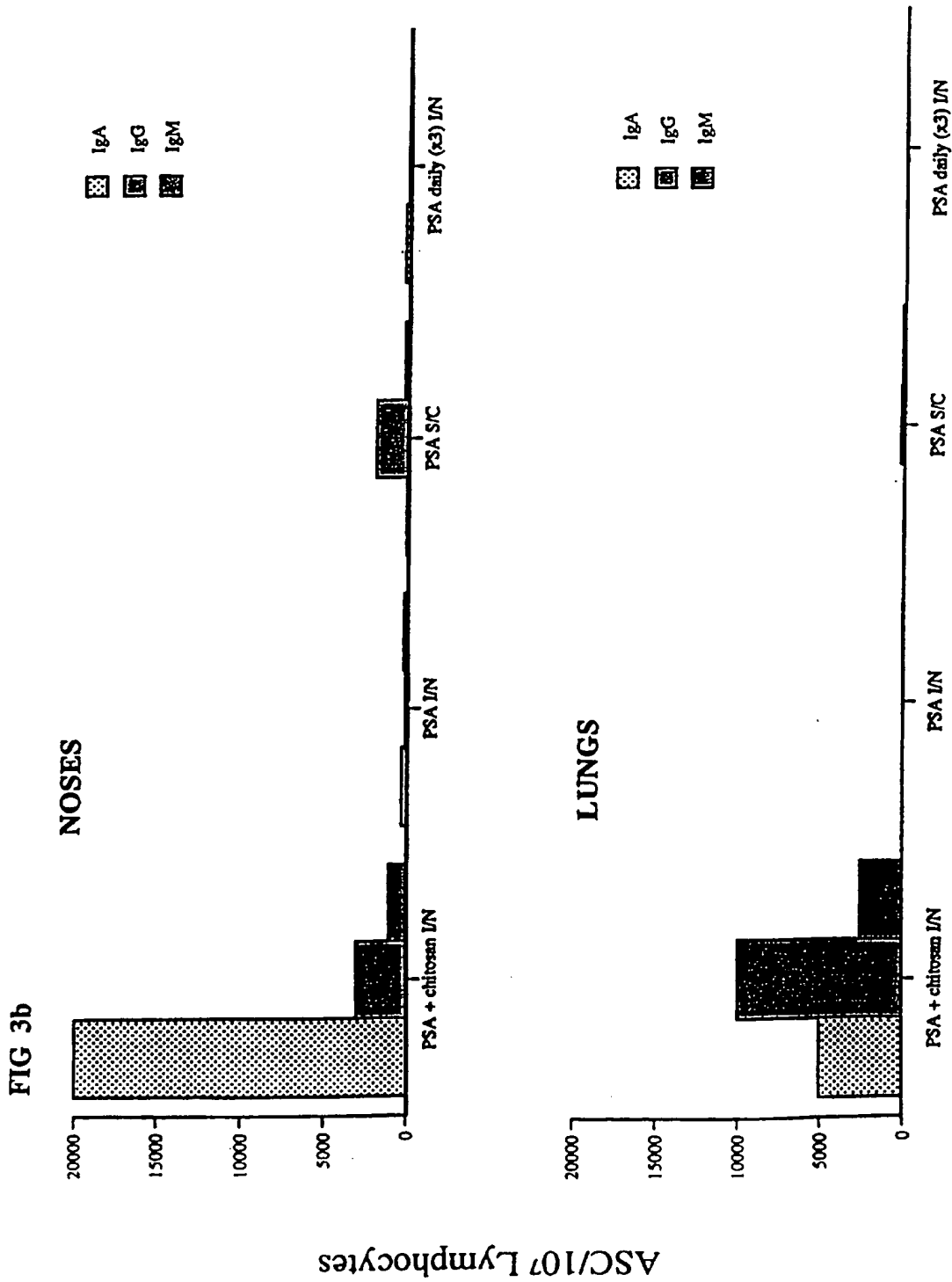
FIG. 2



3/6



4/6



5/6

Figure 4a
Anti-FHA IgG antibodies in serum samples

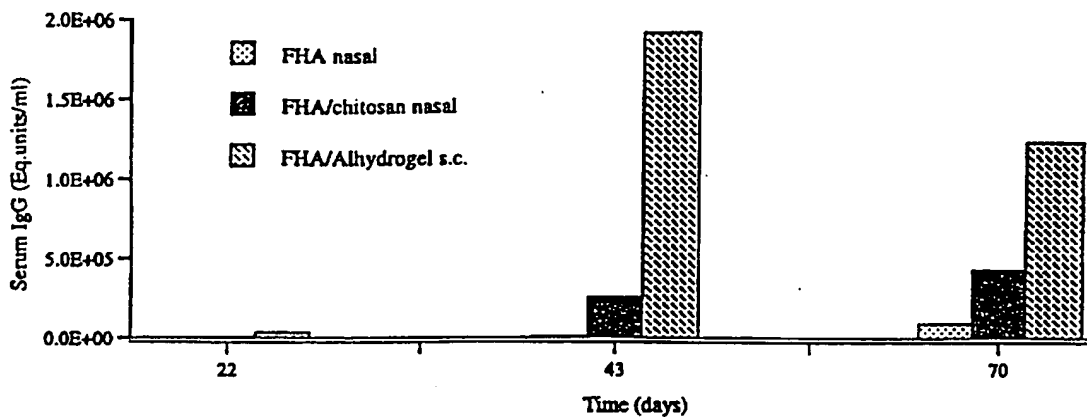


Figure 4b
Anti-FHA secretory IgA antibodies in lung lavage samples

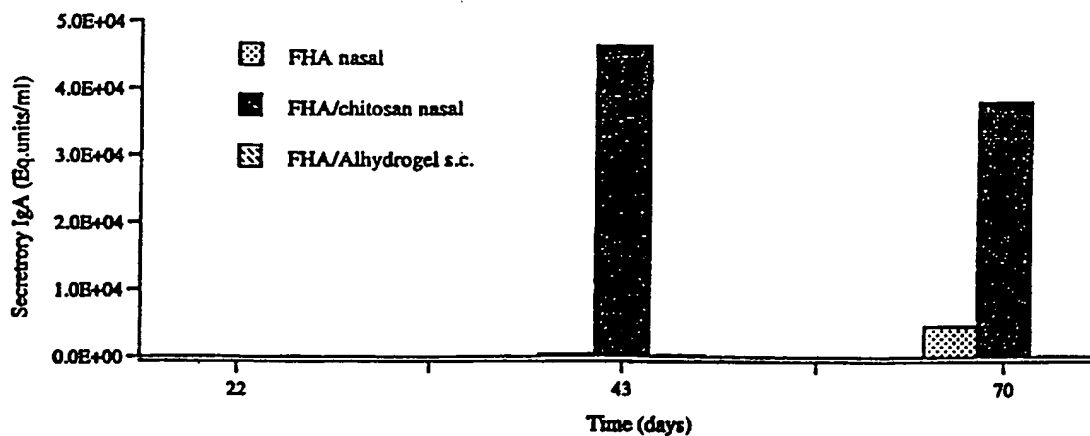
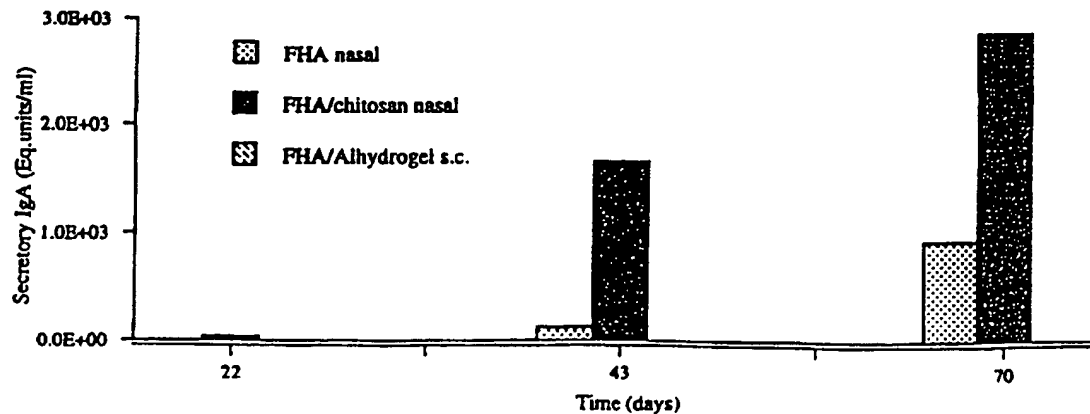


Figure 4c
Anti-FHA secretory IgA antibodies in nasal wash samples



6/6

Figure 5a
Anti-FHA and anti-PT IgG antibodies in serum samples

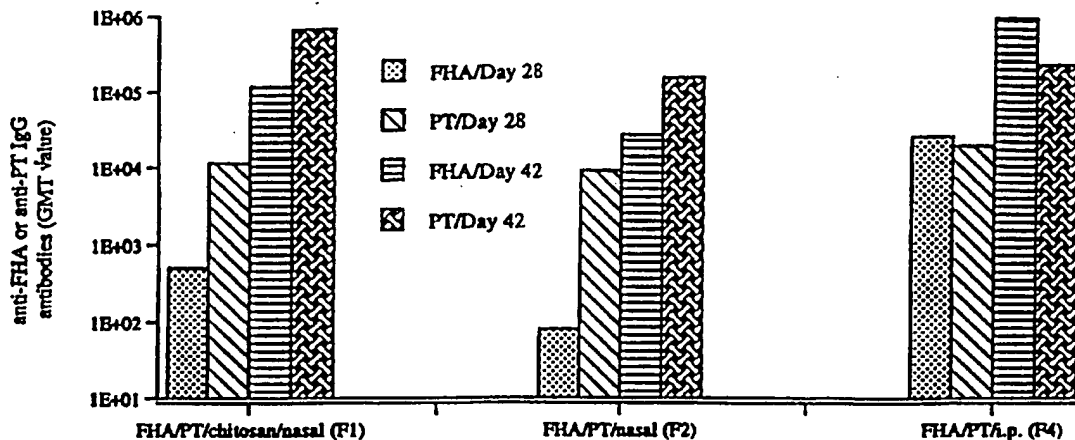


Figure 5b
Anti-FHA and anti-PT secretory IgA antibodies in lung lavage samples

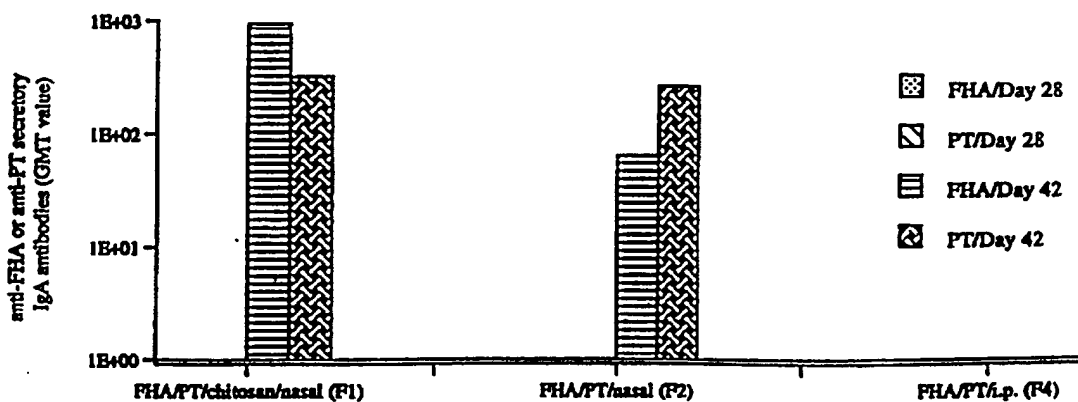
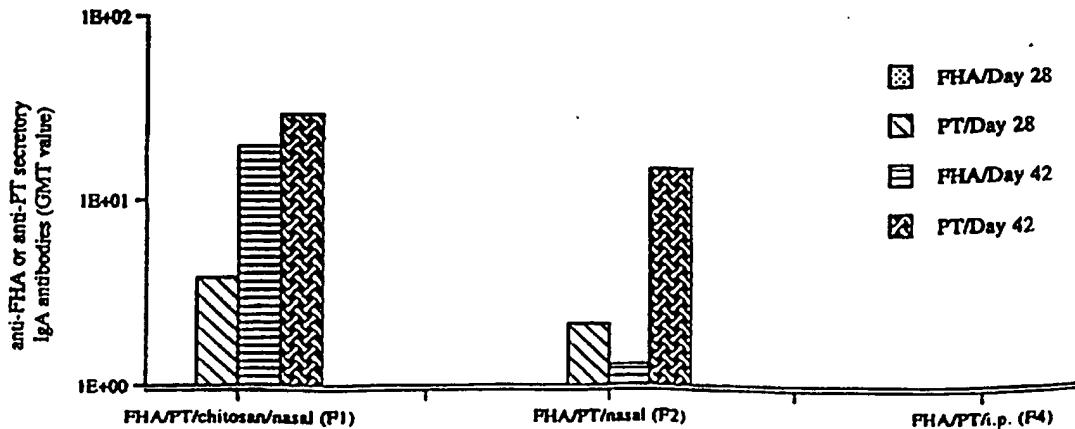


Figure 5c
Anti-FHA and anti-PT secretory IgA antibodies in nasal wash samples



INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/GB 96/03019

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 10421 A (MEDEVA HOLDINGS BV ;CHATFIELD STEVEN NEVILLE (GB)) 11 April 1996 see the whole document ---	1-3,8-27
Y	VACCINE, vol. 12, no. 14, November 1994, pages 1255-1258, XP000612060 OKA T ET AL: "ENHANCING EFFECTS OF PERTUSSIS TOXIN B OLIGOMER ON THE IMMUNOGENICITY OF INFLUENZA VACCINE ADMINISTERED INTRANASALLY" see the whole document --- -/--	1-27

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

27 February 1997

Date of mailing of the international search report

19. 03. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Olsen, L

INTERNATIONAL SEARCH REPORT

Intern. al Application No

PCT/GB 96/03019

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>VACCINE, vol. 13, no. 2, February 1995, pages 155-162, XP000615909 HAAN DE A ET AL: "MUCOSAL IMMUNOADJUVANT ACTIVITY OF LIPOSOMES: INDUCTION OF SYSTEMIC IGG AND SECRETORY IGA RESPONSES IN MICE BY INTRANASAL IMMUNIZATION WITH AN INFLUENZA SUBUNIT VACCINE AND COADMINISTERED LIPOSOMES" see abstract</p> <p>---</p>	1-27
Y	<p>FEMS MICROBIOLOGY LETTERS, vol. 107, 1993, pages 211-216, XP000650962 CAHILL E S ET AL: "MICE ARE PROTECTED AGAINST BORDETELLA PERTUSSIS INFECTION BY INTRA-NASAL IMMUNIZATION WITH FILAMENTOUS HAEMAGGLUTININ" see abstract</p> <p>---</p>	1-27
Y	<p>VACCINE, vol. 3, no. 5, December 1985, pages 379-384, XP002017432 NISHIMURA K ET AL: "ADJUVANT ACTIVITY OF CHITIN DERIVATIVES IN MICE AND GUINEA-PIGS" see the whole document</p> <p>---</p>	1-27
Y	<p>EP 0 183 556 A (IHARA CHEMICAL IND CO) 4 June 1986 see page 7, line 5 - line 11 see page 8, line 5 - line 10 see page 9, line 2 - line 17</p> <p>---</p>	1-27
A	<p>WO 90 09780 A (DANBIOSYST UK) 7 September 1990 cited in the application see page 7, line 19 - line 21</p> <p>-----</p>	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 96/03019

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9610421 A	11-04-96	AU 3526995 A	26-04-96
EP 0183556 A	04-06-86	JP 6015476 B	02-03-94
		JP 61268626 A	28-11-86
		JP 1791018 C	29-09-93
		JP 4081967 B	25-12-92
		JP 61130230 A	18-06-86
		CA 1261264 A	26-09-89
		JP 7023313 B	15-03-95
		JP 62123123 A	04-06-87
		US 4971956 A	20-11-90
WO 9009780 A	07-09-90	CA 2045472 A	26-08-90
		DE 69008346 D	26-05-94
		DE 69008346 T	25-08-94
		EP 0460020 A	11-12-91
		ES 2055904 T	01-09-94
		US 5554388 A	10-09-96